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Clarke B E; Newton S E; Carroll A R; Francis M J; Appleyard G; Syred A D; Highfield P E; Rowlands D J; Brown F. Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. NATURE. (1987 Nov 26-Dec 2) 330 (6146) 381-4.

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Thanks.

EXPRESSION IN YEAST OF AMINO-TERMINAL PEPTIDE FUSIONS TO HEPATITIS B CORE ANTIGEN AND THEIR IMMUNOLOGICAL PROPERTIES

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Hepatitis B core protein (HBcAg) is a potent antigen that gives both a T-cell-dependent and a T-cell-independent antibody response. It has been shown that a foreign epitope can be fused to the amino terminus of HBcAg without affecting particle integrity, and that the resulting chimaeric cores retain the immunogenicity of the foreign epitope. Here we describe the efficient expression in yeast of two different chimaeric cores, carrying epitopes of Foot and Mouth Disease Virus (FMDV) or human chorionic gonadotrophin (hCG), which are candidates for FMD and contraceptive vaccines, respectively. These cores could not be produced in *E. coli* in soluble form but were expressed to high levels in yeast. We constructed a yeast expression vector that allows rapid production of different chimaeric cores by cloning in cassettes encoding foreign epitopes. Both FMDV and hCG-cores were shown to present the epitopes at the surface of the particles. The FMDV-cores produced in yeast were efficient inducers of neutralising antibodies in guinea-pigs after one low dose.

Hepatitis B core antigen (HBcAg), which encapsidates the genome of hepatitis B virus (HBV), is a 21kD protein able to self-assemble into 27nm core particles when expressed in foreign host cells[1,2]. The gene encoding HBcAg is unusual in that it gives rise to two related polypeptides HBcAg and hepatitis B e antigen (HBeAg), depending on which of two initiation codons is used[3]. Initiation at an upstream ATG leads to translation of 'pre-core' sequences encoding a signal peptide. Cleavage of the signal peptide and the C-terminal protamine-like region of pre-HBeAg gives rise to the mature, secreted HBeAg (17kDa). Alternatively, use of the downstream ATG produces the 21kD HBcAg. Expression in *E. coli* and yeast of HBcAg DNA containing 'pre-core' sequences indicated that HBcAg containing N-terminal extensions would still form particles (P. E. Highfield, pers. comm. and ref. 2).

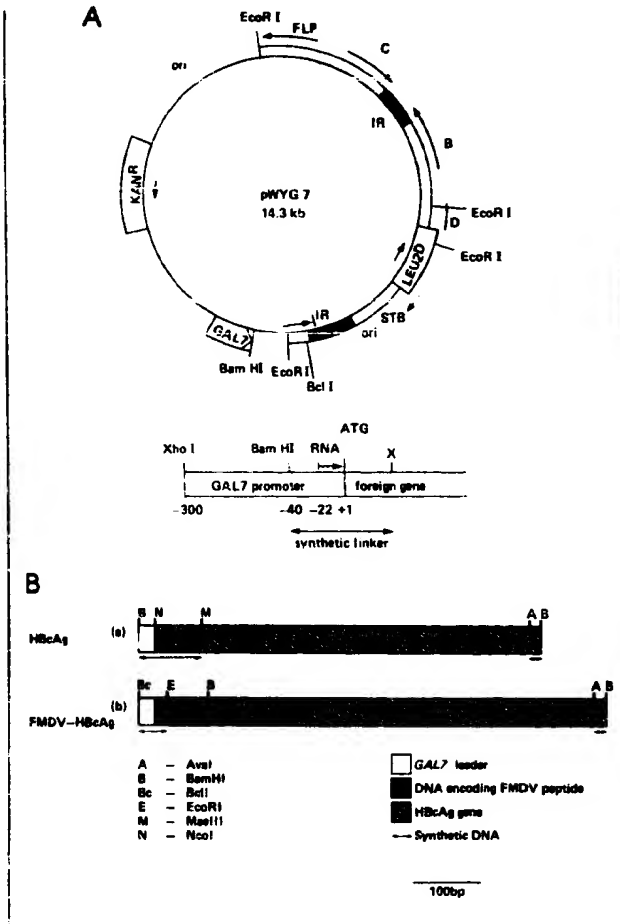
HBcAg particles are exceptionally potent antigens, having both a T-cell-dependent and a T-cell-independent response[4]. This property is probably due to their multivalent particulate nature and to the presence of several efficient helper T-cell epitopes in the polypeptide. Given their ability to form chimaeric particles carrying N-terminal extensions, they have been proposed as a general carrier vehicle for the presentation of epitopes in the development of subunit vaccines. This type of approach for polyvalent antigen presentation was first described by Valenzuela and co-workers[5] who fused a portion of the herpes simplex virus glycoprotein D to the N-terminus of hepatitis B surface antigen. Another example is the use of yeast transposon virus-like particles[6] (TyVLPs).

The first example of the use of HBcAg as a carrier was with the major immunogenic epitope of FMDV, the 142–160 peptide of the capsid protein VP1. A chimaeric particle containing this peptide has been shown to be a potent immunogen, giving rise to protective antibodies in guinea-pigs when injected once in low doses[7]. Several other N-terminal HBcAg fusions, produced using an *E. coli* expression system, are also proving to be highly immunogenic[8]. However, difficulties have been encountered with some HBcAg fusions expressed in *E. coli*. In particular, the FMDV-fusion could not be expressed due to its toxicity to the host cell. Consequently, this protein was expressed from vaccinia virus, a system not designed for high-level protein yield[7]. A further problem associated with occasional HBcAg fusion proteins when expressed in *E. coli* has been their sequestration as insoluble inclusion bodies[8]. Here we describe the efficient expression in yeast of the FMDV-fusion protein, and of a fusion to a peptide derived from human chorionic gonadotrophin (hCG) β-subunit[9], which was insoluble when produced in *E. coli*.

RESULTS

Construction of yeast expression vectors for HBcAg and fusion proteins. We used the vector pWYG7, containing the galactose-regulated *GAL7* promoter, to produce HBcAg and the fusion proteins (Fig. 1). pWYG7 is derived from pJDB219, which contains the entire yeast 2μ plasmid[10], modified to contain a kanamycin-resistance marker from pUC4K[11]. The *GAL7* promoter consists of 260bp of synthesised DNA, which is sufficient to confer full promoter activity[12], with an engineered BamHI site, at −40 relative to the ATG codon, upstream of the normal mRNA initiation site. The 5′ ends of foreign genes are linked into the BamHI site using oligonucleotides, which re-create a native *GAL7* leader upstream of the ATG. The 3′ ends are ligated to the BclI site of 2μ, just upstream of the *FLP* transcriptional terminator[13]. Yeast cells containing pWYG7 or its derivatives can be selected using either G418 resistance or leucine prototrophy. In host cells that are free of native 2μ plasmid (2μ°) the plasmids are extremely stable and will probably not require continued selection on fermenter scale-up. The use of a tightly regulated promoter such as *GAL7* is essential for this stability when expressing foreign genes whose products show any degree of toxicity.

The vaccinia expression vector for FMDV-HBcAg, pvFOHc[7] was used as a source of HBcAg DNA in all



C

fusion

oRI
ATTCAGCTCCGAACCTGCGTGGTGACCTCCAGGTTCTGGCTCAGAAACTTGCTCGTACCCTGCCG
N S A P N L R G D L Q V L A Q K V A R T L P
◄ FMDV peptide ►

BamHI
CTCGGGATCCGCGCGCCCTTGGGTGGCTTTGGGGCATG
A P D P R A L G W L W G M
spacer ►◄ pre-core ►

fusion

oRI
ATTCTACATGTCATCATCCAAGATTTCAAGATTCATCAAGTAGTAAAGCACCACCTCCAAGT
N S T C D D P R F Q D S S S S K A P P P S
◄ hCG peptide

BamHI
CAAGTCGTTCTAGATTGCCAGGACCTTCTGATACACCTATCCTTCCACAGGATCCGCGCGCC
P S P S R L P G P S D T P I L P Q D P R A
►◄spacer►

GGTGGCTTTGGGGCATG
G W L W G M
pre-core ►

FIGURE 1 (A) Map of the yeast expression vector pWYG7 containing a synthesised *GAL7* promoter fragment. Regions of the vector derived from the bacterial plasmid pMB9 are in single lines. 2μ plasmid regions are boxed, and the major features are indicated (inverted repeats IR; FLP, B, C, and D genes; STB locus; origin, ori; the *LEU2* marker). The FLP terminator is indicated by →1. Foreign genes are linked to the BamHI site at the 5′ ends and ligated to BclI at the 3′ end. (B) Expression cassettes for HBcAg and FMDV-HBcAg fusion. (C) Peptide extensions in the FMDV and hCG fusions and the DNA encoding them.

constructions (Fig. 1 for details). The 3′ end of the gene was synthesised from the AvaI site providing a BamHI site downstream of the stop codon. For expression of HBcAg, the 5′ end was synthesised from the MaeIII site and modified to contain an NcoI (CCATGG) site at the initiation codon, and *GAL7* upstream sequences to a BamHI site. For FMDV-HBcAg-expression, the region 5′ to the gene in pvFOHc, from the EcoRI site, was synthesised with the *GAL7* upstream DNA to a BclI site. The expression cassettes for HBcAg (BamHI to BamHI) and FMDV-HBcAg (BclI to BamHI), propagated in the intermediate vectors pKGC and pKGF respectively (not shown), were ligated between the BamHI and BclI sites of pWYG7 in order to generate pWYG7HBC and pWYG7HBF (Fig. 1). The hCG fusion vector was constructed by replacing the EcoRI to BamHI fragment in pWYG7HBF, encoding the FMDV peptide, by an EcoRI-BamHI cassette encoding the 109–145 peptide from the carboxy terminus of hCG β-subunit, generating pWYG7HBG (Fig. 1).

We have been able to use these expression vectors to generate vectors expressing other amino-terminal fusions in one step. Epitopes encoded by EcoRI-BamHI cassettes can be readily inserted into pWYG7HBF in one step in a three-way ligation using the unique XhoI site of pWYG7, as was done in the case of hCG. Otherwise NcoI-NcoI oligonucleotides encoding the epitope can be ligated at the NcoI site of pKGC and the BamHI to BamHI expression cassette then transferred to pWYG7.

Expression of chimaeric proteins. Two chimaeric HBcAg proteins were expressed. First the FMDV-fusion, which contains the major (linear) immunogenic epitope of FMDV as well as the cell attachment site (VP1 peptide 142–160 + spacer peptide)[7]. Second, the hCG-fusion containing the 109–145 peptide, from the carboxy terminus of the hCG β-subunit, which has been proposed as a contraceptive vaccine[9]. This protein could be produced in *E. coli* but in an insoluble form. We also expressed native HBcAg in order to compare the amounts produced in our yeast system with those published previously, and with the two fusion proteins.

When soluble protein extracts from induced yeast (S150-2B 2μ°) cells transformed with pWYG7HBC, pWYG7HBF and pWYG7HBG were compared to extracts from induced, untransformed yeast, novel protein bands of the predicted sizes (21.3, 25.2 and 26.7kD respectively) were detected in Western blots of SDS-polyacrylamide gels (Fig. 2B). All the HBcAg or fusion protein was in the soluble protein fraction (data not shown). Expression levels of HBcAg were quantified by scanning of Coomassie-stained gels (Fig. 2A) or ELISA and found to range from 6 to 10% of soluble cell protein (200–300 mg/l in shake-flasks). Since we found HBcAg ELISA not to be reliable for the FMDV- and hCG-fusion proteins, the expression level of the FMDV-fusion was determined only by scanning Coomassie-stained gels (Fig. 2A), and found to be about 2% (60 mg/l in shake-flasks). The hCG-fusion could not be resolved from yeast proteins and was therefore estimated by comparison to HBcAg in Western blots: the two were expressed at similar levels. We have found other HBcAg fusion proteins that are expressed in yeast to levels of 10% or greater, e.g., a fusion to a 30 amino acid peptide derived from the *Bordetella pertussis* 69K antigen (unpublished).

Particulate nature of the proteins and epitope presentation. To test the particulate nature of the fusion proteins, extracts of soluble proteins were centrifuged through sucrose density gradients and the fractions analysed for HBcAg by ELISA or by reaction with peptide antibody of aliquots dotted onto nitrocellulose. The result

of the FMDV-fusion (Fig. 2D) shows that all the HBcAg-reacting material sedimented near the A_{260} peak (80S ribosomes) indicating full assembly into core particles. Analysis using anti-FMDV peptide serum indicated that all the FMDV-reacting material in gradients co-sedimented with the HBcAg-reacting material (data not shown). A similar analysis showed that the hCG-fusion was also fully assembled. Though the core particles migrate near the ribosome peak, it has been possible to prepare particles of several fusion proteins of greater than 80% purity using a single sucrose gradient purification step (eg hCG-cores, Fig. 2C). However, the FMDV-cores could not be purified to this level (Fig. 2C) using sucrose gradients since there were losses due to aggregation. Epitope presentation on the surface of the FMDV- and hCG-cores was examined by electron immunocytochemistry using colloidal gold probes and the relevant specific antibody. Chimaeric cores bound colloidal gold in the presence of specific antibody (Fig. 3); controls using antibodies of unrelated specificity were negative.

***In vitro* analysis of FMDV-core preparations.** The FMDV-cores were further tested to determine whether the VP1 142–160 peptide was intact. Some of the FMDV-cores, previously produced using the vaccinia virus expression system, had shown cleavage at the amino terminus of the epitope followed by loss of immunogenicity. This may be related to the known proteolytic susceptibility of this peptide in the intact FMD virion[14].

Dilutions of two FMDV-core preparations (A1 and A2) were analysed for HBcAg and FMDV peptide using a double antibody sandwich ELISA. Results showed (Table 1) that the preparations contained equivalent activity against HBcAg and FMDV 142–160 peptide, suggesting that the peptide was present on all HBcAg particles. Furthermore, an anti-peptide MAb, produced to an FMDV 137–162 peptide/B galactosidase fusion protein[15] also reacted to a similar degree with the FMDV-core preparations. Since this MAb recognises a 142–160 synthetic peptide but not 143–160 or other synthetic peptides shortened at their amino termini[16], it appears that the FMDV peptide epitope on FMDV-cores is intact.

Immune response of guinea-pigs to FMDV-cores. Anti-HBcAg, anti-FMDV 142–160 peptide and FMDV neutralizing antibody titres elicited in guinea pigs following inoculation with FMDV-cores are given in Table 2. High levels of anti-HBcAg (3.7 $\log_{10}$) and anti-FMDV peptide (2.4 $\log_{10}$) antibody appeared within 14 days and these continued to rise up to 56 days, when a second inoculation was given. In the presence of very high anti-HBcAg antibody levels (greater than 5.0 $\log_{10}$) the second inoculation had little or no booster effect. However, antibody levels did persist at high levels for the following 28 days, at which time the experiment was terminated. The significant levels of anti-FMDV peptide antibody were reflected in virus neutralizing activity, which appeared at 14 days and increased to protective levels in all four animals (2.2–2.7 $\log_{10}$ SN_{50}) at 56 days[17]. These results with an inoculum containing only 0.65μg of FMDV peptide (6.5μg of FMDV-cores) confirm that the yeast-derived material has the same marked immunogenicity as the material made using a vaccinia virus expression system.

DISCUSSION

We have described the efficient production in a yeast expression system of chimaeric hepatitis B core particles carrying foreign epitopes. The two examples given here are of cores that could not be expressed in a soluble form in *E. coli* yet were expressed efficiently in yeast as soluble

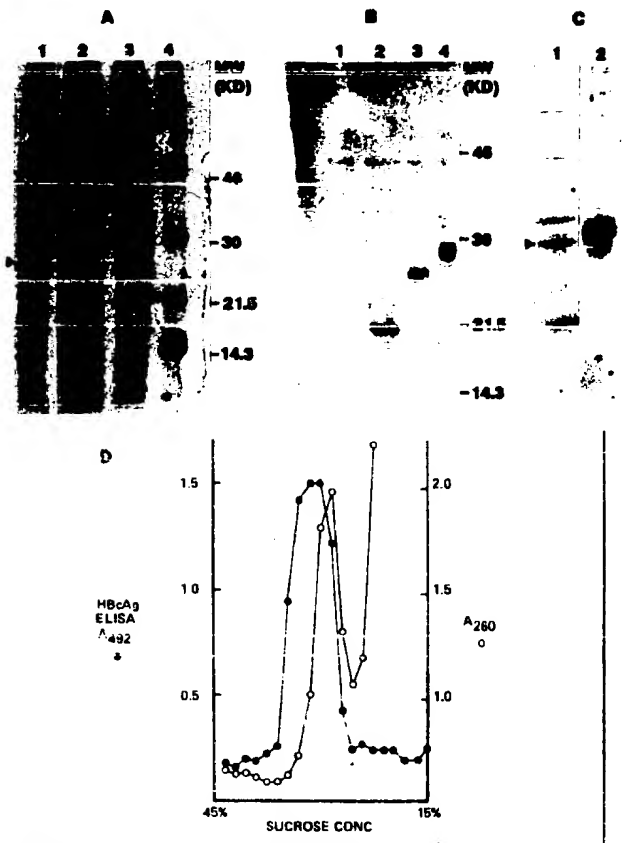


FIGURE 2 Analysis of HBcAg and fusion proteins. (A) Coomassie blue-stained 12.5% SDS-polyacrylamide gel of soluble extracts from induced cells containing pWYG7HBF (track 1), no plasmid (track 2) or pWYG7HBC (track 3). (B) Western blot of similar gel to (A) probed with rabbit anti-HBcAg serum as first antibody. Tracks 1, 2, 3, 4 contained soluble proteins from induced cells containing no plasmids, pWYG7HBC, pWYG7HBF and pWYG7HBG, respectively. (C) Coomassie blue-stained gel of sucrose-gradient purified preparations of FMDV-cores (track 1) or hCG-cores (track 2). (D) Sucrose density gradient centrifugation of soluble yeast extract containing FMDV-cores. Fractions were analysed for HBcAg using ELISA.

TABLE 1 ELISA analysis of FMDV-core samples.

Fusion protein sample	Test antiserum: Anti-HBcAg	Anti-FMDV 142-160 peptide	Anti-FMDV/B gal fusion protein mAb
A1	1.77*	1.97	1.85
A2	2.24	2.26	2.26

*$\log_{10}$ 50% ELISA

TABLE 2 Immune response of guinea pigs to FMDV-cores.

Antibody activity	Days post primary inoculation: 0	14	28	56	63	70	84
Anti-HBcAg	1.0*	3.7	4.6	5.1	4.9	5.3	5.5
Anti-FMDV peptide	1.0*	2.4	3.3	3.8	3.9	3.9	3.7
FMDV neutralization	0.6**	1.1	1.5	2.5	2.5	2.7	2.7
				↑			

↑—second inoculation
*—mean $\log_{10}$ ELISA endpoint titre
**—mean $\log_{10}$ SN_{50} vs 100$TCID_{50}$ of virus

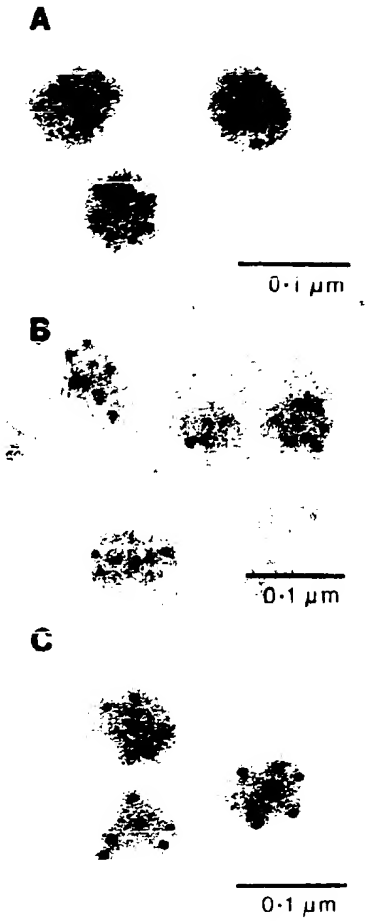


FIGURE 3 Electron micrographs of HBcAg particles (A), FMDV cores (B), and hCG-cores (C) labelled with anti-HBcAg serum (A) or the appropriate anti-peptide serum (B and C). Presence of the foreign epitope on the surface of the cores is demonstrated by antibody binding and immunogold labelling.

proteins. The expression levels obtained, 2% of soluble protein for FMDV-cores and 6–10% for hCG-cores, are equivalent to 60 mg/l and 200–300 mg/l in shake-flasks, respectively. In high-density fermentations, 10 to 20-fold shake-flask density, these figures should yield gram per litre levels.

The levels of native HBcAg produced using our *GAL7* expression vector, 6–10% of cell protein, are comparable to those of Imamura et al.[18], but significantly lower than the 40% level reported by Kniskern et al.[19]. These authors used the constitutive *GAPDH* promoter, which is more active than *GAL7*. The *GAL7* promoter is also known to be limited in multi-copy vectors by a shortage of transcriptional activators, including GAL4 protein[20]. Against these disadvantages, *GAL7* is tightly regulated, an advantage in vector stability where the foreign protein has any toxicity. We are currently examining alternative promoters to see if we can increase expression levels.

The high level of expression of HBcAg is probably partly due to its stability in the yeast cell[19]. Superoxide dismutase (SOD) is another protein that accumulates to very high levels due to its stability[21]. Since SOD fusion proteins are also stable they have been used for the efficient production of a number of foreign proteins. Where a mature protein is required, the SOD fusion can be cleaved at a specific site (e.g., in a proinsulin-SOD fusion[22]). A similar approach has been used in fusions to the yeast transposon Ty virus-like particles (VLPs), for example in the production of HIV TAT protein[23]. The hybrid VLPs, containing a factor Xa cleavage site, are readily purified by sucrose gradient centrifugation, and the mature TAT protein is released using factor Xa. Chimaeric HBcAg particles may also prove to be useful in this way, since we have found that, in general, expression levels in yeast remain high with fusion proteins. This is so even in the case of a much larger fusion, containing a 25kD immunogenic repeat region of *Plasmodium falciparum* circumsporozoite antigen (KMB and M. Lockyer, unpublished).

The FMDV-cores were expressed at a rather lower level than HBcAg, 2% of cell protein. This is not due to less efficient transcription since the FMDV-core transcript is present in yeast cells at the same high levels as that of HBcAg or other fusions (not shown). In *E. coli* the FMDV-fusion was toxic, though no toxicity was detected upon induction of yeast cells. We were only able to recover a proportion of the FMDV-cores since they appeared to aggregate during purification. However, we believe it should be possible to use alternative purification methods to prevent aggregation, since the FMDV-cores are initially fully soluble in the yeast cell lysate. The fact that the immunogenicity of FMDV-cores has been shown to approach that of the virus itself makes them a realistic proposition as a subunit vaccine for FMD. Previously the major limitation had been the low yield of the chimaeric particles produced using a vaccinia virus expression system, but we have shown here that large amounts can be produced in yeast.

Problems associated with the current FMD vaccine include, firstly, antigenic variation of the virus[24], secondly, the requirement of a cold chain for administration of over 2 billion vaccine doses annually, and thirdly, outbreaks of disease associated with incomplete inactivation of virus in vaccines. Our recent data suggest that freeze-dried FMDV-cores are stable at room temperature (B.E.C., unpublished) and, obviously, there is no risk of viral infection from a yeast-derived subunit vaccine. Furthermore, recent work shows that equivalent synthetic FMDV peptides for each of the seven serotypes all give rise to neutralising antibodies[25]. Using the simple cassette replacement system described here it should be relatively straightforward to produce in yeast FMDV-cores for each serotype. A mixture of these cores could have potential as a broad-spectrum FMD vaccine.

The other epitope discussed here, from the carboxy terminus of the β-chain of hCG, is currently being examined in the development of a human contraceptive vaccine[9]. This region of hCG elicits antibodies, effective in blocking pregnancy, which do not cross-react with other hormones. Previous studies have used synthetic peptides from this region coupled to a variety of macromolecules as carriers, of which the most effective that would be acceptable for human use were tetanus and diphtheria toxin. We have recently shown that the immune response against several peptides is more efficient when the peptides are presented on HBcAg particles than when chemically coupled to other carriers[26]. Furthermore, it has been possible to induce immune responses against HBcAg fusions in the complete absence of adjuvant[26].

One general limitation to all peptide and carrier immunogens is that, although high levels of neutralising antibodies may be induced, the memory helper T-cells required for an optimal anamnestic response will be carrier-specific rather than epitope-or pathogen-specific.

Therefore the use of this type of immunogen as a vaccine may be restricted to cases where pre-existing antibody levels alone confer protection against the pathogen or cases where immunity is required for a limited period[27]. Both the FMDV and hCG examples fall into these categories.

Recently, Argos and Fuller[28] modelled the three-dimensional structure of HBcAg on the known X-ray crystallographic structure of the homologous Mengovirus capsid protein VP3. Neither the amino nor carboxy terminus of the polypeptide was on the external surface of the protein in this model. Therefore, if the model is correct, a certain amount of distortion would have to occur to accommodate a peptide extension on the surface. Our results of antibody binding and immunogold labelling of FMDV- and hCG-cores demonstrate the presence of the peptide extension on the particle surface (Fig. 3). Surprisingly, carboxy terminal extensions have also been made, to HBeAg, without disrupting particle assembly, and the added peptides are also external to the particle[29]. It is difficult to compare the immunogenicity of the carboxy terminal to amino terminal fusions since immunisations with the former were carried out using multiple injections with Freund's complete adjuvant. However, it has been shown recently that particulate HBeAg is considerably less immunogenic than HbcAg, HBeAg cores being purely T-cell dependent antigens[30].

EXPERIMENTAL PROTOCOL

Construction of plasmids. The structure of pWYG7 and its derivatives is described in Results. Oligonucleotides for the constructions were synthesised on a Pharmacia Gene Assembler and purified by electrophoresis on a denaturing polyacrylamide gel. Their 5' ends were phosphorylated before ligation. Ligated products were introduced into *E. coli* strain MC1061[31]. The sequences of cloned synthesised regions were confirmed using the chain termination method in M13[32].

Yeast transformation and induction. The expression vectors were introduced into a 2μ° version of the *Saccharomyces cerevisiae* strain S150-2B (*a leu2 his3 ura3 trp1*)[33] using the lithium transformation procedure[34]. Transformed yeast cells were incubated in YPD broth[35] at 30°C overnight prior to plating on selective medium (YP + 2% glycerol + 500 μg/ml G418), in order to increase transformation frequencies. G418 resistant colonies were screened for the Leu^+ phenotype before expression analysis. For induction, transformants were grown to the mid-exponential phase (10^7 cells/ml) in YP + 2% raffinose + 500 μg/ml G418 at 30°C, 2% galactose was added and the culture shaken at 30°C for a further 16–24 h. The cells were then harvested by low-speed centrifugation, washed once in water and resuspended in ice-cold break buffer (20 mM sodium phosphate, pH7.0, 0.1% triton X-100, 4 mM PMSF, 4 mM EGTA, and 2 μg/ml each of pepstatin, antipain, leupeptin and chymostatin; 5 ml for a 250 ml culture). Acid-washed glass beads (0.45 mm) were added and the cells broken by vortexing. The crude cell lysate was cleared by centrifugation at 10,000 g for 15 min and the protein concentration determined using the Bio-Rad protein assay.

Protein analysis. Proteins were separated in SDS-polyacrylamide gels[36] and stained with Coomassie blue or transferred to nitrocellulose filters for immunological detection. Stained gels were scanned using a Joyce-Loebl Chromoscan 3. Western blots were developed using horse-radish peroxidase-conjugated second antibody and 4-chloronaphthol colour reagent. In order to test for particle formation, cell extracts were layered over 15–45% w/v sucrose density gradients in phosphate-buffered saline and centrifuged in a Beckman SW28 rotor for 4 h at 28,000 rpm. The gradient was fractionated and the fractions analysed by ELISA or dot blotting.

Immunonegative stain technique. Core particles were dried onto electron microscope grids and immuno-labelled by sequential incubation with specific antibody diluted with phosphate-buffered (0.01M) saline (0.15M) pH 7.2 containing 1% BSA and either protein A-gold probes, or goat anti-mouse IgG-gold probes (each 10 nm gold spheres; Janssen Biotech, Belgium). After washing with water, the cores were negatively stained with phosphotungstate and examined in a Philips 300 transmission electron microscope.

Enzyme linked immunosorbent assay (ELISA). Modifications of the indirect and double antibody sandwich ELISA techniques described by Voller et al.[37] were used for antigen and antibody assays.

Antigen assay. Microplates were coated overnight at +4°C with rabbit anti-HBcAg IgG. After washing, 0.5 $\log_{10}$ dilutions of FMDV peptide/HBcAg fusion protein were added and the plates incubated for 1 hr at 37°C. Plates were then washed and guinea pig anti-HBcAg or guinea pig anti-FMDV peptide 142–160 or mouse monoclonal antibody (MAb) against the FMDV peptide at predetermined fixed dilutions was added. After a further incubation for 1 hr at 37°C, plates were washed and anti-guinea-pig or anti-mouse IgG-peroxidase conjugates was added. After a final incubation for 1hr at 37°C the plates were washed and an enzyme substrate (0.04% *o*-phenylenediamine + 0.004% hydrogen peroxide in phosphate/citrate buffer) was added. The resulting colour development was stopped with 12.5% sulphuric acid after a few minutes and the absorbance at 492 nm was measured in a Titertek Multiskan (Flow Laboratories, Irvine, Ayrshire). The A_{492} values obtained were plotted against the $\log_{10}$ reciprocal fusion protein dilution and a 50% endpoint titre was calculated by reference to minimum and maximum A_{492} values.

Antibody assay. Microplates were coated overnight at +4°C with bacterially expressed HBcAg or synthetic FMDV peptide 141–160. Plates were washed and test guinea pig serum samples at 0.5 $\log_{10}$ dilutions from 1:10 were added. After incubation for 1 hr at 37°C, plates were washed and anti-guinea pig IgG-peroxidase conjugate was added. After a further hour at 37°C plates were developed with substrate solution as described above. The A_{492} values obtained were plotted against the $\log_{10}$ reciprocal antiserum dilution and the antibody endpoint titres were calculated by reference to a negative standard (a 1:10 dilution of pre-inoculation guinea pig serum).

Neutralization assay. The FMDV neutralizing activity of serum samples against 100 $TCID_{50}$ of virus was determined using a micro neutralization test with IBRS-2 cells[38]. Each test was performed in duplicate and the results were recorded as the mean $\log_{10}$ reciprocal of the serum dilution that gave confluent cell sheets in 50% of the microplate wells (SN_{50}).

Animals. Four female Dunkin-Hartley guinea pigs weighing approximately 450g were inoculated intramuscularly with a 6.5μg dose of HBcAg fusion protein, containing 0.65μg of FMDV peptide sequence, formulated in incomplete Freund's adjuvant (IFA). All animals were boosted with a similar inoculum at 56 days. Serum samples, collected at 7 to 14 day intervals, were stored at −20°C prior to analysis for antibody activity.

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